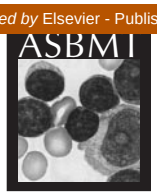
Biology of Blood and Marrow Transplantation 8:429-434 (2002)



Effective Purging of Autologous Hematopoietic Stem Cells Using Anti–B-Cell Monoclonal Antibody–Coated High-Density Microparticles Prior to High-Dose Therapy for Patients with Non-Hodgkin's Lymphoma

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Received May 24, 2001; accepted September 26, 2001

ABSTRACT

Contamination of hematopoietic stem cells (HSCs) with tumor cells has been associated with increased incidence of relapse in patients with non-Hodgkin's lymphoma following autologous HSC transplantation. Effective purging of tumor cells may improve the results of HSC transplantation, but current methods of purging are technically difficult to perform with large numbers of cells and do not consistently remove all detectable cells. We report a pilot clinical trial in which 10 patients with relapsed B-cell non-Hodgkin's lymphoma received high-dose chemotherapy followed by infusion of autologous HSCs depleted of B-cells by high-density microparticles (HDM) coated with anti-CD19 and anti-CD20 monoclonal antibodies (BCell-HDM). HSCs were mobilized with cyclophosphamide and granulocyte colony-stimulating factor. In 6 of the 10 patients, B-cells were detectable by immunocytochemical analysis of the apheresis products prior to treatment. Following treatment with the BCell-HDM, no B-cells were detected in the products from 5 of these patients, a result representing a median depletion of >2.2 logs (range, >0.4 to >5.1 logs). The median recovery of nontarget cells postdepletion was 73% for $CD34^+$ cells and 78% for $CD3^+$ cells. All patients received high-dose cyclophosphamide, BCNU (carmustine), and etoposide prior to reinfusion of their B-cell–depleted autologous HSCs. The median number of $CD34^+$ cells cryopreserved was 3.6×10^6 cells/kg (range, 2.2-10.1 $\times 10^6$ cells/kg). Engraftment was rapid in all cases, with a median time to achieve an absolute neutrophil count of 0.5×10^9/L of 10 days (range, 8-11 days). The median time to achieve a platelet count of 20×10^9/L unsupported by platelet transfusion was 11.5 days (range, 8-17 days). This nonmagnetic negative-depletion technology is simple, rapid, and effective in depleting target cells to undetectable levels, with excellent recovery of nontarget cells.

KEY WORDS

Autologous transplant • B-cell lymphoma • Purging

INTRODUCTION

High-dose therapy with hematopoietic stem cell (HSC) transplantation has become recognized as an effective therapy in patients with relapsed non-Hodgkin's lymphoma (NHL) [1-3]. Approximately 40% of those patients whose disease is still responsive to therapy have long-term disease-free survival after high-dose therapy and autologous HSC transplantation. A randomized prospective trial showed that the combination of high-dose therapy and autologous bone marrow transplantation (BMT) is superior to conventional-dose salvage chemotherapy in treating patients with relapsed diffuse aggressive lymphoma [1]. Studies in patients with

indolent lymphomas also suggest that a subset of patients experience long-term disease-free survival following high-dose therapy with autologous stem cell support [4-11].

An important consideration in autologous HSC transplantation is the potential for contamination of the stem cell product by reinfusion of tumor cells. Studies involving genetic marking of autologous marrow prior to infusion suggest that contaminating tumor cells contribute to relapse in patients with neuroblastoma, acute myeloid leukemia, and chronic myeloid leukemia [12,13]. Additional evidence has come from studies employing high-dose chemoradiotherapy followed by reinfusion of anti–B-cell monoclonal antibody–purged autologous bone marrow in patients with B-cell NHL [5,6,14]. The detection of lymphoma cells by polymerase chain reaction (PCR) in the treated bone marrow following ex vivo bone marrow purging was a highly significant predictor of subsequent relapse. Conversely, the absence of residual lymphoma cells detectable by PCR in the reinfused marrow was strongly associated with continuing complete remission. Additional indirect evidence that residual tumor cells contribute to relapse has been shown in aggressive lymphomas, in which the ability to culture malignant cells from the harvested stem cell product was highly predictive of subsequent relapse following high-dose therapy [15]. Current technology for purging autologous stem cell products of lymphoma cells has limitations. Techniques that select $CD34^+$ progenitor cells have been shown to reduce the number of tumor cells in the autograft by approximately 3 logs [16,17]. However, an increased incidence of infection posttransplantation has been described, presumably due to the removal of normal immune effector cells from the graft [18,19]. A combination of multiple monoclonal antibodies and rabbit complement can deplete approximately 2 to 4 logs of tumor cells, albeit with some variability [20]. However, this technology is time-consuming and relies on nonstandardized biological reagents. It is also difficult to apply to peripheral blood samples because of the high cell numbers harvested compared to bone marrow samples. Immunomagnetic beads with anti–B-cell monclonal antibodies have also been used to deplete neoplastic cells from stem cell collections [21,22]. More recently, in vivo purging of B-cells with rituximab administered prior to stem cell mobilization and apheresis has been reported [23,24]. Here we report the use of a new negative-depletion technology that uses high-density microparticles (HDM) made of nickel and coated with anti–B-cell monoclonal antibodies (BCell-HDM) to deplete B-cells from autologous peripheral blood stem cell (PBSC) preparations prior to transplantation in patients with relapsed B-cell NHL [25-28]. The objectives of the pilot study were to evaluate the extent of B-cell depletion and recovery of $CD34^+$ cells and $CD3^+$ T-cells in the treated autologous PBSCs, to evaluate engraftment following infusion of the HDM-treated PBSCs, and to evaluate infusional toxicity of the HDM-treated PBSCs. Treatment of PBSC collections with the BCell-HDM was associated with high recovery of $CD34^+$ cells and T-cells and >2 logs of depletion of B-cells. Moreover, reinfusion of the treated cells was safe, and engraftment was prompt. This rapid and effective approach for depleting malignant B-cells will permit the undertaking of further clinical trials to evaluate the role of ex vivo purging of tumor cells prior to autologous transplantation.

MATERIALS AND METHODS

Selection of Patients and Treatment Protocol

Patients were eligible for this study if they were younger than 65 years, had B-cell NHL as defined in the National Cancer Institute Working Formulation or Revised European-American Lymphoma (REAL) classification, and had relapsed after standard chemotherapeutic regimens [29,30]. Lymphoma cells of eligible patients had to demonstrate CD20 and/or CD19 expression confirmed by results of flow cytometric or immunhistochemical analysis as described [31]. Patients had to have sensitive disease and achieve minimal disease status defined as lymph node masses less than 5 cm in greatest diameter and histologic evidence of bone marrow involvement of 20% or less of the intratrabecular space as determined by iliac crest biopsy. Additional criteria for entry included the absence of comorbid disease of the heart, kidney, lung, and liver and a Karnofsky score above 80%. Informed consent was obtained from all patients. High-dose chemotherapy consisted of the CBV regimen of cyclophosphamide, BCNU (carmustine), and VP-16 (etoposide). The dosages were as follows: cyclophosphamide 750 mg/m^2 twice daily intravenously (IV) on days –6, –5, –4, and –3; BCNU 112.5 mg/m^2 once daily IV on days –6, –5, –4, and –3; and VP-16 200 mg/m^2 twice daily IV on days –6, –5, –4, and –3, as described [32]. The cryopreserved PBSCs were thawed and infused rapidly through a central venous catheter on day 0. Posttransplantation granulocyte colony-stimulating factor (G-CSF) was given at a dosage of 5 μg/kg per day subcutaneously beginning on day +1, until the absolute neutrophil count (ANC) exceeded 1.0×10^9/L. Supportive care was provided as previously described [5].

PBSC Mobilization and Collection

PBSCs were mobilized using cyclophosphamide, 3 g/m^2 infused IV over 2 hours, followed 1 day later by G-CSF, 10 μg/kg subcutaneously daily until completion of apheresis. Leukapheresis components were collected when the ANC was $>1.0 \times 10^9$/L. Three total blood volumes were processed each day (for a maximum of 4 days) until a minimum of 2×10^6 $CD34^+$ cells/kg had been isolated after processing with the BCell-HDM Cell Separation System. For the first 2 patients enrolled, an additional apheresis component containing at least 1×10^6 $CD34^+$ cells/kg was collected and cryopreserved without purging for use in the event of delayed engraftment or nonengraftment of the BCell HDM-treated PBSC.

Treatment of PBSC with BCell-HDM

BCell-HDM consists of vials of high-density microparticles (HDM) covalently bound to murine monoclonal antibodies recognizing CD19 (CD19-HDM) and CD20 (CD20-HDM). One vial of CD19-HDM and 1 vial of CD20-HDM are required for each depletion cycle. BCell-HDMs are a component of the Eligix Cell Separation System, currently manufactured by BioTransplant Inc (Charlestown, MA). A magnet is used in the system to hold the HDM pellet in place during chamber inversion but is not required for cell separation.

All procedures were performed using sterile technique in a class II type A biosafety cabinet according to the manufacturer's instructions. For each procedure, the HDM were

introduced into 4 sterile disposable 225-mL conical tubes, hereafter referred to as depletion chambers, and washed with normal saline. The leukapheresis product was washed once in normal saline supplemented with autologous plasma and resuspended in normal saline at a final volume of 220 mL. The washed product was added to the first depletion chamber and gently agitated to suspend both the HDM particles and cells. The cells and HDM were then placed on a rotator that gently and constantly inverted the depletion chamber in an end-over-end fashion, permitting the HDM to constantly settle through the cell suspension and collide with target cells. After 5 minutes, rotation was stopped and the depletion chamber was left upright on the rotator for an additional 5 minutes, allowing the HDM particles to settle by gravity. A magnet was used to retain BCell-HDM and captured $CD19^+$ and $CD20^+$ cells, and the cells remaining suspended in the chamber were aseptically transferred to the next depletion chamber. A total of 4 depletion cycles were performed for each component. Following the fourth depletion, the depleted stem cell product was transferred to a sterile final particle capture chamber, secured to the HDM magnet, and placed on the HDM rotator for 5 minutes to capture any residual HDM not retained in the third chamber used. The cells were then washed 3 times and resuspended in autologous plasma. After removal of samples for the required assays, the B-cell–depleted component was then cryopreserved using controlled-rate freezing and stored in vapor-phase liquid nitrogen until required for use.

Analysis of B-Cell Purging Efficiency

An immunocytochemical assay for detection of rare events was modified to detect total $CD20^+$ B-cells in the PBSC products pre– and post–B-cell processing [33]. Briefly, mononuclear cells were isolated by Ficoll-Hypaque separation (Pharmacia, Upsala, Sweden), washed twice in Leibovitz L-15 medium (GIBCO/BRL, Grand Island, NY) supplemented with 10% fetal bovine serum, and resuspended to a concentration of 1×10^7 cells/mL; 3×10^6 cells were placed on slide cytopreparations and immunostained. Cytopreparations were fixed with paraformaldehyde/methanol, washed thoroughly in Dulbecco's phosphate-buffered saline, and incubated with murine monoclonal antibody, antihuman CD20 (Beckman Coulter, Hileah, FL). Alkaline phosphatase immunostaining was performed as previously described. Slides were evaluated by one reviewer, and the total number of $CD20^+$ cells per slide was recorded. The limit of detection of this assay was approximately 1 $CD20^+$ cell per 10^6 nucleated cells.

Recovery of Nontarget Cells

$CD34^+$, $CD3^+$, $CD4^+$, and $CD8^+$ cells were measured by 2-color flow cytometry using a Coulter Epics XL flow cytometer. Samples were removed from the PBSC products prior to and following depletion. These samples were then diluted in 1 mL of autologous plasma and stored at 2°C to 8°C until assayed. For the first 3 patients, $CD34^+$ cells were enumerated by determining the percentage of $CD45^{dim}$ cells that were also $CD34^+$. For the subsequent 7 patients, absolute counting methodology was used [34]. The remaining pre- and postdepletion samples were stained with CD3–fluoroscein isothiocyanate (FITC)/CD19-phycoerythrin (PE), CD3-FITC/CD4-PE, and CD3-FITC/CD8-PE (Beckman Coulter); prepared using the Q-prep device; fixed with 1% formalin solution; and analyzed by standard flow cytometric methods.

Patient Characteristics

Total no. of patients	**15**
Inadequate stem cell collection	**5**
NHL histology at ASCT (n = 10), no. of patients	
Follicular center cell grade I or II	**2**
Diffuse large B-cell lymphoma*	**7**
Mantle cell lymphoma	**1**
Median age at transplantation (range), y	**53.5 (38-64)**
Bone marrow involvement with NHL at ASCT	**3**
Prior therapy	
Median no. of chemotherapy regimens (range)	**3 (1-6)**
No. of patients with prior radiation therapy	**2**

*Includes 2 patients with a history of indolent lymphoma whose disease transformed to aggressive histology lymphoma.

Monitoring Levels of Nickel Murine Immunoglobulin and Human Antimouse Antibody

Serum and urine samples for determination of nickel concentration were collected prior to PBSC infusion and at 24 hours and 1 week after infusion. Nickel concentration was measured by atomic absorption (Galbraith Laboratories). The limit of detection of the assay was 14.4 μg/mL. Enzyme-linked immunosorbent assays were used to test the pre- and postdepletion apheresis products for the presence of murine immunoglobulin (Ig) (an IgG1 assay to detect anti-CD19 and an IgG2a assay to detect anti-CD20). The limit of detection of the assay was 0.78 ng/mL. Testing for human antimouse antibody (HAMA) response (Dianon Systems, Stratford, CT) was performed on patient serum at screening, at day +100, and 6 and 12 months posttransplantation. The limit of detection of the assay was 74 ng/mL.

RESULTS

Patient Characteristics

Fifteen patients with recurrent B-cell NHL received mobilization chemotherapy. PBSC mobilization was adequate in 10 patients, who then received high-dose therapy followed by infusion of BCell-HDM–modified PBSCs. The characteristics of these 10 patients prior to autologous stem cell transplantation (ASCT) are detailed in the Table. The median age of these patients was 53.5 years (range, 38-64 years). Histological analysis results indicated diffuse large B-cell lymphoma in 7 patients, follicular lymphoma grade I/II in 2 patients, and mantle cell lymphoma in 1 patient. Prior to mobilization all patients were in a minimal disease state, with 3 patients having overt histologic bone marrow involvement.

Analysis of Ex Vivo Stem Cell Treatment

Ten patients underwent apheresis and had adequate PBSC mobilization. Seven of these 10 patients required only a single apheresis to reach the required $CD34^+$ target number of 2×10^6/kg; 2 patients required 3 aphereses, and

1 patient required 4 aphereses. Six patients had B-cells detectable by immunohistochemical analysis of the predepletion product, 3 patients had no detectable B-cells, and 1 patient was unevaluable because of high background staining. Following cell processing with the BCell-HDM, no B-cells were detected in any of the apheresis products from 5 of these 6 initially positive patients. One patient had detectable B-cells in both pre- and postdepletion products. The mean log reduction of B-cells was >2.4 logs (median, >2.2 logs; range, >0.4 to >5.1 logs). The pre- and postdepletion products were examined by flow cytometry for numbers of $CD34^+$, $CD3^+$, $CD4^+$, and $CD8^+$ cells. The median recoveries of $CD34^+$, $CD3^+$, $CD4^+$, and $CD8^+$ cells postdepletion were 80%, 81%, 74%, and 72%, respectively. These results were not corrected for losses due to cell sampling.

Because murine antibodies are a component of the BCell-HDM, we examined postdepletion apheresis products in all patients for the presence of murine antibodies, and none were detected in any products from the 10 patients.

Reinfusion of BCell-HDM–Treated Cells and Engraftment

Toxicities related to infusion of BCell-HDM–treated product occurred in 3 patients. The toxicities were mild and consistent with sequelae associated with infusion of unmanipulated products cryopreserved with dimethylsulfoxide. Grade 1 toxicities included nausea, vomiting, dyspnea, cough, altered taste, flushing, tachycardia, and hypotension. One patient had grade 3 hypertension. No nickel levels above background were detected in any serum or urine samples in any patient after PBSC reinfusion. No patient had a detectable nickel allergic response. HAMA levels were assessed prior to treatment and at 100 days, 6 months, and 12 months following ASCT. No patient had a detectable HAMA response following PBSC reinfusion. The median time to achieve an ANC of 500/μL was day +10 (range, day 8 to day 11) and the median time to reach a platelet count of 20,000/μL unsupported by platelet transfusion was day +11.5 (range, day 8 to day 17). Durability of engraftment at day +30 posttransplantation was demonstrated in all patients.

Treatment Outcome

Of the 10 patients treated, none suffered early treatment-related deaths. As of March 28, 2001, 5 patients had relapsed, at 2.5, 8.5, 19.3, 19.5, and 22.6 months. The other 5 patients remained alive and in complete remission with a median follow-up of 16 months (range, 7 to 20.5 months). The Kaplan-Meier estimate of patients alive and disease-free at 16 months is 79% (90% confidence interval, 57%-100%). Only 1 patient died, at 16 months posttransplantation, following relapse at 2.5 months. Of the 5 patients who relapsed, 4 relapsed in sites of prior disease and 1 in both prior and new sites.

DISCUSSION

A major question regarding the use of autologous or peripheral blood as sources of stem cells following high-dose therapy is whether reinfused tumor cells may in fact contribute to relapse in these patients. Upwards of 70% of patients with follicular NHL have histologic evidence of bone marrow involvement at diagnosis and virtually all patients with relapse have bone marrow positive for tumor cells at the time of relapse [35]. In a study from our institution, all patients had bone marrow involvement, detectable by PCR assay for the bcl-2 translocation, at the time of bone marrow harvest, even if the bone marrow appeared histologically negative for tumor cells [36]. Similar evidence for the presence of PCR-detectable occult disease in the presence of histologically normal marrow has been reported from other centers [37-41]. In light of the studies suggesting that reinfused tumor cells contribute to relapse [12,13], several approaches have been taken to provide a tumor-free stem cell preparation. These approaches include immunologic and pharmacologic techniques to purge malignant cells from the graft, as well as steps to enrich for normal hematopoietic progenitors through CD34 selection [4-6,8,10,14,31,42,43]. Although the primary source of stem cells has largely shifted from bone marrow to peripheral blood, there is no evidence that peripheral blood is a tumor cell–free source of hematopoietic stem cells [44]. Techniques that preserve stem cell numbers but effectively deplete neoplastic cells may address the contribution of tumor cell contamination to relapse following high-dose therapy.

One of the problems of current positive and negative selection techniques is the loss of normal progenitor and effector cells. This pilot study demonstrated that the BCell-HDM Cell Separation System used to deplete B-cells from autologous PBSCs was not associated with any delay in engraftment of neutrophils or platelets. The median times to reach an ANC of 500/μL and a platelet count of 20,000/μL were similar to those expected from infusion of the same number of $CD34^+$ cells in an unmanipulated autograft [45].

The BCell-HDM cell separation device yielded a B-cell depletion of greater than 2.4 logs (range, >0.4 to >5.1 logs). The measurement of the extent of target cell depletion is highly dependent on the number of target cells in the predepletion apheresis product as well as the limit of detection of the assay used to quantify the target cells. Four of the 10 patients in this study had no detectable B-cells in the predepletion product, possibly because of the use of cyclophosphamide in the mobilization regimen. Furthermore, of these 4 patients, 1 patient had been treated with rituximab prior to enrollment in the study, and 1 patient had received fludarabine, agents known to reduce the number of circulating B-cells and total lymphocytes, respectively.

One of the problems of CD34 selection is the potential loss of both progenitors and other effector cells [46]. In the present study there was limited impact on the recovery of nontarget cells including $CD34^+$ and $CD3^+$ cells. Recovery of $CD34^+$ cells is important because of the correlation between the number of $CD34^+$ cells infused and the speed and durability of engraftment [45]. Recovery of $CD3^+$ cells is important because delays in lymphoid reconstitution may predispose patients to severe opportunistic infections, such as cytomegalovirus [18,19]. This predisposition to infection has been reported following the use of CD34 selection prior to ASCT in an attempt to minimize the risk of reinfusion of potentially contaminating tumor cells.

One concern in this pilot trial was toxicity due to the infusion of cells treated with a device consisting of murine monoclonal antibodies conjugated to nickel particles. We observed

no significant infusional toxicity that could be ascribed to the use of the BCell-HDM Cell Separation System. Moreover, we found no evidence of in vivo exposure to nickel or murine monoclonal antibody, nor did any patient develop an HAMA response after infusion of the treated PBSCs.

In summary, use of the BCell-HDM Cell Separation System resulted in marked reduction of B-cells without significant impact on recovery of nontarget cells. Treatment of PBSCs did not appear to alter the rate of neutrophil and platelet recovery posttransplantation. Future studies using this device will be focused on comparing target B-cell depletion, target cell recovery, and engraftment in a randomized trial and ultimately determining the impact on disease-free survival of purging PBSCs prior to autologous transplantation in B-cell NHL.

ACKNOWLEDGMENTS

This work was supported by NIH grant CA 66996.

We are indebted to the nurses, adult oncology fellows, and social workers of the Dana-Farber Cancer Institute and the housestaff and nursing staff of the Brigham and Women's Hospital for their excellent care of these patients. We also thank the staff of the Dana-Farber Cancer Institute Blood Donor Center and the Connell and O'Reilly Families Cell Manipulation Core Facility for the care taken in the collection and processing of the PBSC.

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